

DNA melting within a binary σ^{54} -promoter DNA complex

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SUMMARY

The σ^{54} subunit of the bacterial RNA polymerase requires the action of specialised enhancer binding activators to initiate transcription. Here we show that σ^{54} is able to melt promoter DNA when it is bound to a DNA structure representing the initial nucleation of DNA opening found in closed complexes. Melting occurs in response to activator in a nucleotide hydrolysing reaction and appears to spread downstream from the nucleation point towards the transcription start site. We show that σ^{54} contains some weak determinants for DNA melting that are masked by the Region I sequences and some strong ones that require Region I. It seems that σ^{54} binds to DNA in a self-inhibited state, and one function of the activator is therefore to promote a conformational change in σ^{54} to reveal its DNA melting activity. Results with holoenzyme bound to early melted DNA suggest an ordered series of events in which changes in core to σ^{54} interactions and σ^{54} -DNA interactions occur in response to activator to allow σ^{54} isomerisation and the holoenzyme to progress from the closed complex to the open complex.

INTRODUCTION

Accessing the information in DNA often relies upon the action of DNA binding proteins that are able to generate non canonical B-DNA structures. Recombination, replication, methylation, repair and transcription are processes that proceed through intermediates where DNA is distorted. The process of RNA transcript formation by all RNA polymerases must involve a DNA melting event to reveal the template DNA strand (1-3). Distortion of the DNA leading to the nucleation of strand separation occurs within the closed complex formed between RNA polymerases and the promoter. Following isomerisation of the polymerase, full stable DNA opening is evident, which is thought to have spread from the initial nucleation site. Single strand DNA-binding activities in the RNA polymerase are required for DNA opening and, in the case of the bacterial RNA polymerase, the sigma subunit plays an important role (1, 3-7). For the σ^{70} type factor, binding to the core enzyme induces conformational changes in a single stranded DNA binding region of the protein. As a consequence of these conformational changes, σ^{70} gains specificity for the non template strand of the melted region in the open complex (8, 9). For the σ^{54} type factor, unrelated by sequence to σ^{70} , the determinants of single strand DNA binding are less well described. Single strand DNA binding by σ^{54} is however evident (4, 10, 11). The sequences that σ^{54} recognises as single strand DNA are between the -12 promoter element and the start site (4). Importantly, the activity of the σ^{54} -holoenzyme is tightly regulated at the DNA melting step, but promoter binding to form the initial σ^{54} -holoenzyme closed complex is not highly regulated (12).

The closed complexes formed with the σ^{54} -holoenzyme are silent for transcription unless acted upon by an enhancer binding activator protein (13-15). A network of protein and DNA interactions involving σ^{54} function to maintain a stable holoenzyme conformation that rarely changes spontaneously to allow DNA melting and transcript initiation (11, 16-22). The conformationally restricted closed promoter complex isomerises to an open promoter complex, in which the DNA strands are melted out, in a reaction where the activator consumes ATP or another nucleoside triphosphate (14). As a part of this reaction pathway, σ^{54}

contributes to the creation of a local structural distortion within the closed complex (23). σ^{54} binds tightly to the distorted promoter DNA and can be shown to isomerise independently of the core RNA polymerase in a reaction that has all the remaining requirements for open complex formation (4, 24). Isomerisation is associated with an increased DNase I footprint of σ^{54} on DNA, extending towards the transcription start site (24).

Here we use DNA footprinting to show that the DNA within the isomerised σ^{54} -DNA complex has melted, and that some melting is negatively regulated by the Region I of σ^{54} . However, extensive melting requires Region I. Additionally, changed interactions between σ^{54} and the nucleated DNA are evident in complexes where melting has occurred. We show that the presence of core RNA polymerase inhibits those changes in σ^{54} -DNA interactions that occur in response to activator, consistent with the view that tight binding to the early melted DNA limits DNA opening (4). Results provide clear evidence in favour of an activation mechanism in which conformational changes in a basal σ^{54} -DNA complex are brought about by the enhancer binding activator. Activator independent melting suggests that the activator does not function exclusively as a site specific DNA helicase for DNA opening (11, 18, 22).

EXPERIMENTAL PROCEDURES

DNA and proteins

The promoter fragments used in this work are *Escherichia coli glnHp2* from -60 to +28, in which T at -13 was replaced by G and the -11/-10 sequence replaced with a CA/TG mismatch to create a short unpaired DNA element next to a consensus GC (-13/-12) promoter element or mismatched sequence between -11 and -6 (see Fig. 1). *Sinorhizobium meliloti nifH* promoter fragments from -60 to +28 containing either a CA/TG mismatch immediately adjacent to the consensus GC element or the A-12 top strand base missing (gapped duplex) were also used (see Fig. 1, ref. 24). Synthetic -60 to +28 DNA strands were annealed to create the duplex, with either strand 5'-³²P kinased. The unlabelled strand was at 2 fold molar excess.

The *Klebsiella pneumoniae* σ^{54} protein, its Region I deleted derivative lacking the first 56 amino acids ($\Delta I\sigma^{54}$) and Region I (amino acids 1-56) were prepared as described previously (11, 25). Activator was *E. coli* PspF lacking a functional C-terminal DNA binding domain (PspF Δ H₁TH, 26). *E. coli* core RNA polymerase was from Epicentre Technologies.

DNA binding assays

End labelled DNA (16-100 nM) plus 1 μ M σ^{54} or $\Delta I\sigma^{54}$ in a 10 μ l reaction in STA buffer (25 mM Tris-acetate pH 8.0, 8 mM Mg-acetate, 10 mM KCl, 1 mM DTT and 3.5% (w/v) PEG 8000) were incubated for 5 minutes at 30°C. Activator PspF Δ H₁TH (0.5-4 μ M), and dGTP or GTP or GTP γ S (4 mM) were added for a further 10 minutes. Region I was at 0.5 μ M. Where indicated, heparin (100 μ g/ml) was added for 5 minutes prior to gel loading. Free DNA was separated from sigma bound DNA on 4.5% native polyacrylamide gels run in 25 mM Tris/200 mM glycine at room temperature.

DNA footprints

Binding reactions were conducted as above, footprinting reagents added, reactions terminated and the bound and unbound DNA separated on native gels as above. DNA was then excised, processed and analysed on a denaturing 10% polyacrylamide gel. For DNase I

footprints, 1.75×10^{-3} units of enzyme (Amersham Life Sciences) was added to a 10 μ l binding reaction for 1 minute followed by addition of 10 mM EDTA to stop cutting. For KMnO_4 footprinting, 4 mM fresh KMnO_4 was added for 30 seconds followed by 50 mM β -mercaptoethanol to quench DNA oxidation. Gel isolated DNA was eluted into 0.1 mM EDTA, pH 8.0 (DNase I footprints) or H_2O (KMnO_4 footprints) overnight at 37°C . KMnO_4 oxidised DNA was cleaved with 10% v/v piperidine at 90°C for 20 minutes. Recoveries of isolated DNA were determined by dry Cerenkov counting and equal numbers of counts loaded onto gels.

RESULTS

Previously we showed that purified σ^{54} bound to the *Sinorhizobium meliloti nifH* promoter was able to isomerise if the DNA template had an unpaired sequence downstream of the GC element of the promoter (24). The isomerisation also required activator and nucleoside triphosphate hydrolysis, and was characterised as an extended σ^{54} -DNA interaction towards the transcription start site. The unpaired DNA downstream of the GC promoter element was suggested to mimic the nucleation of DNA melting (early melted DNA) seen in σ^{54} closed complexes, which normally requires σ^{54} and core RNA polymerase (23). Here we have used variants of the σ^{54} dependent *E. coli glnHp2* promoter (27, Fig. 1) to explore DNA melting by σ^{54} . We chose to generate a nucleated *glnHp2* promoter because the high AT content of the sequence should facilitate the detection of unstacked T residues using KMnO_4 as a DNA footprinting reagent (28). Base unstacking occurs when DNA melts, and the associated increased reactivity to KMnO_4 is readily detected.

Isomerisation of the σ^{54} -*glnHp2* complex

Initially we used a gel shift assay to show that σ^{54} bound to the modified *glnHp2* promoter with unpaired DNA at -11/-10 and, in a reaction requiring hydrolysable nucleoside triphosphate (dGTP) and activator, produced a supershifted heparin resistant complex (ss σ -DNA, Fig. 2A, lane 4). As seen before, the same mobility supershifted complex formed with activators of different molecular weights, providing evidence that activator was not stably associated with the isomerised complex (24, and data not shown). Formation of this complex required σ^{54} Region I (Fig. 2A, compare lanes 4 and 8). Addition of Region I *in trans* to $\Delta\text{I}\sigma^{54}$ resulted in a new species with similar mobility to the supershifted complex, independently of activator and nucleotide (Fig. 2A, lanes 9, 10). Using DNase I footprinting, we showed that the DNA within the σ^{54} isomerised complex was further protected than in the complex forming with σ^{54} in the absence of activator and nucleotide (Fig. 2B). The downstream edge of the σ^{54} footprint extended to about -5 (Fig. 2B, lane 3). In the isomerised complex, the footprint extended clearly to +2, but a partial footprint to +5 was also detected (Fig. 2B, lane 4). The upstream edge of the footprint was not easily discernible due to background DNA

fragments in the undigested sample (Fig. 2B, lanes 1 and 5). Binding of the $\Delta\text{I}\sigma^{54}$ does not lead to the extended DNase I footprint of σ^{54} seen with the activator dependent isomerised complex but weakly footprints to about -7 (Fig. 2B, lanes 9 and 10). Addition of Region I *in trans* to $\Delta\text{I}\sigma^{54}$ resulted in a DNase I footprint indistinguishable from that of the non-isomerised σ^{54} -DNA complex (Fig. 2B, compare lanes 7 and 8 with 3).

Overall results show that σ^{54} -DNA interactions at *glnHp2* are changed by the action of activator in a nucleotide dependent manner. Non-hydrolysable nucleotide GTP γ S did not substitute for dGTP to produce an extended footprint (data not shown). Qualitatively, results are similar to those obtained at the *S. meliloti nifH* promoter (24), and clearly indicate that activator dependent σ^{54} isomerisation may be readily demonstrated at a number of different promoters.

Activator dependent DNA melting by σ^{54}

We used KMnO_4 to probe for DNA melting within isomerised complexes. KMnO_4 footprints at 30°C using the *S. meliloti nifH* early melted DNA (-12/-11) in the isomerised complex did not convincingly show extra DNA melting but the unpaired T at -12 of the heteroduplex region was much less reactive in both the σ^{54} -DNA complexes (data not shown). These KMnO_4 footprints were repeated at 37°C and with a promoter derivative having a single base pair of heteroduplex at -12 (top strand A replaced with C, ref. 24). Results with the -12/-11 heteroduplex showed no extra DNA melting at the elevated temperature but the unpaired T at -12 in the isomerised complex was more reactive to KMnO_4 (data not shown). However, footprints using the -12C heteroduplex DNA in the isomerised complex showed that at 37°C the template strand T-9 had a 2-fold increase in KMnO_4 reactivity indicating some extra DNA melting (data not shown). This contrasts results obtained with *glnHp2* derivatives (see below, Fig. 3) where considerable extra DNA melting in the isomerised complex is seen at 30°C. It seems melting of the *nifH* promoter within the isomerised complex occurs less frequently than with *glnHp2* and may relate to differences in the ease with which the DNA strands of the two promoters can separate (see discussion).

Footprints of the *glnHp2* -11/-10 promoter DNA show convincingly that the isomerised

complex has extra DNA melting. As seen in Fig. 3A, σ^{54} strongly protects the template strand unpaired T at -11 from KMnO_4 attack (compare lanes 2 and 3). In the isomerised complex this protection is lost, and KMnO_4 reactivity is evident at the unpaired T at -11, as well as at new positions -9 and -7 (Fig. 3A, compare lanes 3 and 4). The bases at -11, -9 and -7 must be within an altered DNA structure compared to the free DNA and the non isomerised σ^{54} -DNA complex. It seems that activator brings about some extra DNA melting as well as changing the interaction of σ^{54} with the T at -11. The KMnO_4 reactivity of the bands was quantified to enhance the reliability of the interpretation, see Table 1. The same patterns of enhanced reactivity and protection were seen in three independent experiments. Controls in which either activator or hydrolysable NTP were omitted, or a non-hydrolysable NTP ($\text{GTP}\gamma\text{S}$) was used, showed that the extra DNA melting at -9 and -7 and the changed footprint at -11 required activator plus hydrolysable nucleotide (data not shown). In the absence of these components, the σ^{54} -DNA footprint remained unchanged and the only complex evident in the gel shift assay was the fast running σ^{54} -DNA complex (data not shown).

KMnO_4 was used to probe the non template strand in σ^{54} -DNA and isomerised σ^{54} -DNA complexes forming with the modified *glnHp2* promoter. As shown in Fig. 3B (see also Table 1), T residues at -8 and -6 were KMnO_4 reactive in isomerised complexes that formed in response to activator and hydrolysable nucleotide (lane 4). In the absence of activator and hydrolysable nucleotide, the same sequences were no more reactive to KMnO_4 than the naked DNA (Fig. 3B compare lanes 2 and 3, see also Table 1). As seen for the template strand (Fig. 3A), isomerisation of the σ^{54} -DNA complex is accompanied by some local DNA melting. In both cases melting seems to extend from the -11/-10 heteroduplex region by at least (interpretation is limited by the placement of a potentially reactive T) an extra 4 base pairs towards the transcription start site (summarised in Fig. 7). Melting at these locations is seen in natural open promoter complexes forming with the σ^{54} -holoenzyme (13-15, 29). The lack of reactivity of non template T -4 suggests that either the transcription start site sequence is not stably opened in the isomerised complex, or that T -4 is protected by σ^{54} from KMnO_4 attack.

Weak deregulated DNA melting by σ^{54} lacking Region I

The amino terminal 50 amino acids of σ^{54} (Region I) function to inhibit isomerisation of the RNA polymerase holoenzyme as well as to promote enhancer responsiveness (30-33). Activities of Region I include contributions to the DNA binding function of σ^{54} , particularly the recognition and creation of the nucleated DNA near -12, and an interaction with core RNA polymerase (4, 16, 23, 31, 34). Removal of Region I results in activator independent transcription if the DNA is transiently opened and allows the holoenzyme to engage with pre-melted DNA (11, 18, 20, 25). Using KMnO_4 to probe the template strand of the *glnHp2*- $\Delta\text{I}\sigma^{54}$ complex, we found some evidence for weak activator independent melting. As shown in Fig. 3A (lane 8) the T at -9 shows some increased KMnO_4 reactivity in the $\Delta\text{I}\sigma^{54}$ complex compared to the zero protein control (lane 2). This region of extra DNA melting appears to be a subset of that found within the activator dependent complex forming with full length σ^{54} (compare lanes 4 and 8). The restricted pattern of melting at -9 seen with $\Delta\text{I}\sigma^{54}$ was independent of nucleotide and activator (compare lanes 7 and 8). Quantitative treatment of the KMnO_4 reactivity (Table 1) showed a constant increase in the reactivity of T-9 when $\Delta\text{I}\sigma^{54}$ bound. The increase was seen in three independent experiments. The T at -11 in the $\Delta\text{I}\sigma^{54}$ complex was slightly more protected compared to the isomerised σ^{54} complex but significantly more reactive than σ^{54} alone to KMnO_4 attack (Fig. 3A, compare lanes 8, 3 and 4, also see Table 1). It seems that removal of Region I partially deregulates σ^{54} melting activity. Region I supplied *in trans* did not result in a significant change in KMnO_4 reactivity to suggest a shift in the footprint towards that of the non-isomerised σ^{54} -DNA complex (Fig. 3A, compare lanes 5 and 3 and Table 1). It seems that although Region I binds to the $\Delta\text{I}\sigma^{54}$ -12,-11 complex, this does not lead to large changes in KMnO_4 reactivity (24).

KMnO_4 was used to probe the non template strand in the $\Delta\text{I}\sigma^{54}$ -DNA complexes forming with the modified *glnHp2* promoter. As shown in Fig. 3B (lanes 5-8) and Table 1, no sequences were significantly more KMnO_4 reactive than in the non bound DNA (Fig. 3B lane 2). For the $\Delta\text{I}\sigma^{54}$ -DNA complex, no further changes in KMnO_4 reactivity were detected in response to activator and hydrolysable nucleotide (Fig. 3B, lane 7). Region I supplied *in trans* to $\Delta\text{I}\sigma^{54}$ DNA binding assays did not change non template strand KMnO_4 reactivity (lanes 5 and 6).

Interactions of σ^{54} with DNA pre-opened from -11 to -6 and a gapped structure

The extra DNA opening from -9 to -6 seen in the activator-dependent isomerised complex (Fig. 3) could arise from activator functioning as a DNA helicase. To explore this issue we wished to learn if pre-opening the DNA from -11 to -6 would allow the σ^{54} to bind promoter DNA in an isomerised state without activator. The interaction of σ^{54} with *glnHp2* promoter DNA mismatched from -11 to -6 (see Fig. 1) to mimic the DNA opening seen in the activator dependent isomerised complexes was examined in the presence and absence of activator. Gel shift assays showed that σ^{54} bound the -11 to -6 opened DNA to give an initial complex ($\text{ss}\sigma^{\ast}$ -DNA) with reduced mobility compared to the -11 to -10 opened DNA complex (Fig. 4, compare lanes 2 and 5). The reduced mobility was similar to that of the activator dependent isomerised complex forming on the -11 to -10 opened DNA (Fig. 4, compare lanes 3 and 5). The mobility of the σ^{54} complex with DNA opened from -11 to -6 was unchanged by activator and hydrolysable nucleotide (Fig. 4, compare lanes 4 and 5). To more fully understand the properties of these slow running complexes, we probed them by DNase I and KMnO_4 footprinting. Using *glnHp2* promoter DNA mismatched from -11 to -6, σ^{54} gave a short DNase I footprint to -5 and no extra KMnO_4 reactivity, and footprints were insensitive to activator and nucleotide (data not shown). We conclude that the reduced mobility of the σ^{54} complex with DNA opened from -11 to -6 is due to the altered DNA conformation, and that pre-opening the DNA does not drive the change in σ^{54} needed for the extended downstream DNase I footprint to at least +2 seen in activator dependent isomerised complexes (Fig. 2B, lane 4, ref. 24). Rather a change in σ^{54} conformation driven by the activator seems to be necessary for the extended footprint to +2. The insensitivity of the σ^{54} complex on the -11 to -6 opened DNA to activator suggests that the DNA from -9 to -6 should be in a double stranded form for activator to act on σ^{54} , consistent with prior work with the *S. meliloti nifH* promoter (24).

We previously observed with the *S. meliloti nifH* promoter that removal of the top strand -12A resulted in a σ^{54} -DNA complex that did not form a new slow running species when incubated with activator and hydrolysable or non hydrolysable nucleoside triphosphate (Fig.

5A, compare lanes 2, 4 and 5 with 6, ref. 24). The $\Delta\sigma^{54}$ formed a slower running complex when Region I was *in trans* (Fig. 5A, compare lanes 2, 6 and 7). To characterise these complexes, and to determine their relationship to the isomerised complex, we used DNase I and KMnO_4 footprinting. The *S. meliloti nifH* promoter -12 gap (top strand) probe (see Fig. 1) gave a DNase I footprint with a distinct region that was cut poorly compared to the intact probe (data not shown). This region centred over the -12 gap, and extended about 4 bases either side, suggesting a locally altered DNA structure refractory to DNase I cutting. When σ^{54} bound, extra cutting from -10 to -1 was also evident, suggesting σ^{54} stabilised a double stranded DNA structure otherwise absent from the non bound gapped DNA (data not shown). There was no difference in the DNase I footprint under activating conditions (data not shown). The effects of activating conditions were then gauged by KMnO_4 footprinting. When σ^{54} binds the gapped duplex the single stranded T at -12 (template strand) is protected from attack by KMnO_4 and a modestly increased reactivity to KMnO_4 is seen at T-9 indicative of some activator and nucleotide independent DNA melting (Fig. 5B, compare lanes 2 and 3). The same pattern was seen in the presence of activator and nucleotide (compare lane 3 with lanes 5-7) without evidence for extra activation dependent melting. This suggests a mismatch at -12 is needed for activator response. The slow running $\Delta\sigma^{54}$ complex with Region I *in trans* footprinted like wild type σ^{54} (Fig. 5B, compare lanes 3 and 8), whereas the $\Delta\sigma^{54}$ complex showed less KMnO_4 reactivity at T-9 (compare lanes 8 and 9). This suggests Region I stabilises the melted DNA at -9. The DNA melting seen with the gapped DNA when bound by σ^{54} (Fig. 5B) is consistent with the proposed role of the -12 nucleotide in restricting melting prior to activation (4). The gapped DNA allows melting within the σ^{54} -DNA complex that is not evident with homoduplex DNA (Fig. 5B and data not shown).

Interactions of σ^{54} -holoenzyme with early melted DNA

The early melted DNA structure just downstream of the promoter GC that enables σ^{54} isomerisation is believed to exist in closed promoter complexes, but is apparently absent in the activator dependent open complex (23, 24). The chemical reactivities at -12/-11 seen in closed complexes are not evident in open complexes, rather new melting is evident nearer the

transcription start site (23). To explore the activator responsiveness of σ^{54} -holoenzyme on the *S. meliloti nifH* and *E. coli glnHp2* early melted DNA we conducted gel shift and footprinting assays. As noted earlier, σ^{54} -holoenzyme bound to the early melted DNA assumes a complex with greater resistance to heparin than does the holoenzyme complex on homoduplex DNA (31). This has been suggested to involve a changed interaction between σ^{54} and core RNA polymerase, since the binding of σ^{54} to core RNA polymerase in the absence of early melted DNA is heparin sensitive (31, 34). Gel shift assays with either the *S. meliloti nifH* (Fig. 6A) or the *E. coli glnHp2* (data not shown) early melted DNA in the presence of σ^{54} -holoenzyme showed that under activating conditions no new slow running holoenzyme-DNA complex was detected (Fig. 6A, compare lanes 6 and 7). A reduction in σ^{54} -holoenzyme ($E\sigma$) concentration leads to increased formation of the supershifted σ^{54} -DNA complex ($ss\sigma^{54}$ -DNA, Fig 6A compare lanes 2-6) in the presence of activator and nucleotide, reflecting the non core bound σ^{54} .

We next used DNase I and $KMnO_4$ footprinting to characterise the σ^{54} -holoenzyme-DNA complexes on the early melted DNA and learn if they had isomerised. Results showed that under non activating conditions the isolated σ^{54} -holoenzyme complex gave a short DNase I footprint to -5 and no extra $KMnO_4$ reactivity (data not shown). The footprints were essentially as for σ^{54} except that some extra protection from DNase I by σ^{54} -holoenzyme upstream of -34 was observed with the *E. coli glnHp2* promoter (data not shown). Next we footprinted the σ^{54} -holoenzyme-DNA complexes under activating conditions. The isolated holoenzyme complexes gave footprints indistinguishable from those obtained under non activating conditions, suggesting that the core bound σ^{54} was not able to isomerise efficiently (data not shown). Further experiments with the use of initiating nucleotide (GTP) to potentially stabilise complexes on opened DNA through allowing initiation or with the omission of the heparin challenge to help preserve unstable complexes failed to produce σ^{54} -holoenzyme footprints in which activator dependent changes were evident (data not shown). We conclude that the σ^{54} does not isomerise efficiently when bound to core RNA polymerase in assays using early melted DNA probes.

We confirmed (data not shown) using *S. meliloti nifH* -60 to +28 bp homoduplex DNA

that the heparin resistant open promoter complexes formed by the action of activator and GTP had extended DNase I footprints to at least +13, definition of an exact end point being limited by the resolution of the gel and fragment size to +28 (14, 20, 35). However the efficiency of activator dependent stable complex formation was low in this assay. To address the issue that σ^{54} -holoenzyme bound to the early melted DNA might form new activator dependent complexes (not distinguished from the activator independent complexes because of the common property of heparin resistance) but with low efficiency we also used KMnO_4 as a probe of isomerisation events. Here, isomerisation would be evident as increased reactivity to KMnO_4 rather than protection from DNase I (see Fig. 3A, lane 4). Unlike the activator and nucleotide dependent complexes forming with σ^{54} and the early melted *glnHp2* promoter DNA (Fig. 3A), σ^{54} -holoenzyme did not respond to activator to yield detectable extra melting or any associated loss of KMnO_4 reactivity of the *glnHp2* heteroduplex sequence (data not shown). Overall results show that the holoenzyme complex in contrast to σ^{54} is poorly if at all responsive to activation conditions when bound to the early melted DNA. As discussed later, this may relate to unusually stable complex formation between the early melted DNA and the σ^{54} -holoenzyme.

Core RNA polymerase binding to isomerised σ^{54} -DNA

Having shown that σ^{54} bound to early melted DNA forms an isomerised complex (24 and this paper) but that σ^{54} -holoenzyme does so inefficiently if at all (see above), we conducted an experiment to determine if the conformation of the isomerised σ^{54} -DNA complex allowed core RNA polymerase binding. In this assay the isomerised complex was formed using an end labelled -35 to +6 bp *S. meliloti nifH* -12/-11 DNA fragment (24) in excess of σ^{54} to diminish the amount of free σ^{54} and ensure core RNA polymerase interactions were potentially largely with the DNA bound σ^{54} . Isomerisation reactions were carried out and stopped by adding GDP (Fig. 6B, lane 2 and ref. 24). Then increasing amounts of core RNA polymerase (E) were added to bind σ^{54} -DNA complexes. As shown in figure 6B the addition of increasing amounts of core RNA polymerase (lanes 3-8) depleted the σ^{54} -DNA complex and in parallel an increasing amount of the σ^{54} -holoenzyme bound to the DNA ($\text{E}\sigma$ -DNA) was detected. The

amount of isomerised σ^{54} -DNA complex (ss σ^{54} -DNA) remained relatively constant throughout the titration with core RNA polymerase. It seems that core RNA polymerase preferentially binds the non isomerised σ^{54} -DNA complex. At high core RNA polymerase concentrations (in excess of 0.6 μ M, Fig. 6B, lanes 7 and 8) some core bound to DNA was detected and this contributes to the apparent increased amount of σ^{54} -holoenzyme bound (graphed in Fig. 6C) since core-DNA and holoenzyme-DNA complexes were not fully resolved. We infer that weak binding of the isomerised σ^{54} -DNA complex by core RNA polymerase is because the interface between core and σ^{54} and DNA has changed upon isomerisation. This leads to the suggestion that movements in σ^{54} and DNA are concerted with some in core RNA polymerase for forming the natural open promoter complex, and is discussed below.

DISCUSSION

DNA melting by σ^{54}

Activator dependent isomerisation of σ^{54} results in the spreading of DNA melting away from the nucleated DNA structure and towards the transcription start site (see Fig. 7). A structure melted over at least 6 base pairs is generated. Interactions with the nucleated DNA located at -11/-10 are lessened in the isomerised complex, compared to the initial σ^{54} -DNA complex. Although removal of Region I sequences allows some weak DNA melting by sigma, this is not as extensive as activator driven isomerisation. A comparison of results obtained with the $\Delta I\sigma^{54}$ and intact σ^{54} show that the full DNA melting activity of σ^{54} has some determinants outside of the regulatory Region I (for weak melting at -9), and some that depend upon Region I (for extra melting from -8 to -6).

Isomerised *nifH* promoter- σ^{54} complexes failed to show KMnO_4 reactivity as extensively as *glnHp2* (Fig. 3 and data not shown). If this reflects less melting (as opposed to shielding from KMnO_4), differences in intrinsic DNA opening rates between the two promoters in combination with the sequence independent single-stranded DNA binding activity in σ^{54} (36) could contribute. DNA opening at -9 seen in natural *nifH* open complexes but weakly detected in isomerised complexes with σ^{54} might therefore reflect a stabilising contribution from the core enzyme (37). Clearly changes in the σ^{54} -DNA relationship seen in DNase I footprints of isomerised complexes where melting may not have occurred is consistent with the idea that activator changes σ^{54} structure and pre-opening the DNA does not drive this change (22, 24).

Comparisons of the closed and the activator dependent open σ^{54} -holoenzyme promoter complexes using KMnO_4 and *ortho*-copper phenanthroline footprinting suggest the structure of the DNA immediately downstream of the GC element differs between these complexes and that contact to the -13G is also altered (14, 15, 23). These observations are fully consistent with the lessened sigma interaction at -11 detected by the KMnO_4 footprints of isomerised complexes reported here (Fig. 3). Based on the known interaction between Region I and core polymerase (34, 38), we suggested that the silencing interactions associated with the -13 to -11 σ^{54} -DNA contact (4) can contribute significantly to inhibiting RNA polymerase

isomerisation through restricting conformational changes within the core subunits (16). Changes in promoter DNA and σ^{54} -holoenzyme conformation are probably coupled through σ^{54} Region I to maintain either the closed or the open state of the promoter. Open promoter complexes that form in deregulated transcription by the σ^{54} -holoenzyme are unstable compared to activator dependent open complexes (18, 19, 25). Their instability may be related to incomplete DNA melting beyond -9 as a consequence of the mutations in Region I of σ^{54} , and a need for Region I to stabilise melted DNA (Fig. 5B).

Role of Activator

Results with DNA mismatched from -11 to -6 across the sequences melted in the isomerised σ^{54} -DNA complex support the view that activator drives a conformational change in the σ^{54} rather than generating isomerisation by solely creating an opened DNA structure for σ^{54} binding. For both transcription and for σ^{54} isomerisation pre-opening of the DNA through heteroduplex formation does not by pass activator requirements (22), unless the structure recognised by σ^{54} at -11 is destroyed (36). Rather activator dependent conformational changes in σ^{54} and holoenzyme seem necessary for open complex formation and σ^{54} isomerisation (24, 36). DNase I footprinting shows that σ^{54} clearly binds to double stranded DNA ahead of the locally melted DNA or the fork junction that forms next to the GC promoter element in closed complexes (this work, 4, 24). This suggests that the further melting of the double stranded DNA within the σ^{54} -DNA complex is an active process, in the sense that it is not a primary result of a domain of σ^{54} translocating along the duplex and trapping DNA strands at a fraying fork junction. Energy for duplex destabilisation may come from the tight binding of σ^{54} to the initially locally melted DNA. An activator driven change in σ^{54} -DNA interaction might release σ^{54} from binding to the double stranded DNA downstream of the -12 GC and switch σ^{54} to a conformation that then allows it to bind single stranded DNA. Combined with the single strand DNA binding activities in σ^{54} (4, 36) and the core RNA polymerase (39), σ^{54} isomerisation would lead to formation of the stable open promoter complex.

Stable holoenzyme binding

When bound to core RNA polymerase activator dependent isomerisation of σ^{54} was not evident, in marked contrast to its efficient isomerisation without core. It seems that the short sequence of heteroduplex downstream of the GC inhibits isomerisation of the σ^{54} within the holoenzyme, since on linear DNA activator dependent isomerisation of the holoenzyme is evident (11, 23, 39). The activator dependent movement of σ^{54} across the sequence opened downstream of the GC implied by the results of our KMnO_4 footprints may be inhibited when σ^{54} is bound by core RNA polymerase. This suggests that isomerisation of σ^{54} within the holoenzyme may normally require that the local DNA opening next to the GC does not strongly persist. In the homoduplex the DNA can base pair again, but not in the heteroduplex. Consistent with this view is the observation that the local DNA distortions downstream of the GC and present in the closed complex are apparently changed in the open complex, as judged by the reduced sensitivity of the DNA to two chemical probes of DNA structure (23). The spread of melting observed in the sigma isomerisation assays would then normally be associated with a changing of the DNA structure believed to be locally melted in the closed complex, achieved through a breaking of DNA contacts and a re-binding of σ^{54} to DNA. These considerations suggest an ordered series of events in which changes in core RNA polymerase to σ^{54} interactions and σ^{54} -DNA interactions occur in response to activator to allow σ^{54} isomerisation and the holoenzyme to progress from the closed complex to the open complex. This view is consistent with activator interacting with both core RNA polymerase and σ^{54} (24, 40) to achieve isomerisation of the holoenzyme and with the view that the promoter sequences around the GC contribute to preventing the holoenzyme from isomerising prior to activation and set the target of the activator (4, 24).

Core-Sigma interactions and DNA melting

Results of core RNA polymerase binding with the isomerised and non isomerised σ^{54} -DNA complexes strongly suggest that some points of interaction between σ^{54} and core are changed upon isomerisation of the σ^{54} -DNA complex. The poorer core binding of the isomerised complex likely correlates with the changes in protease sensitivity of σ^{54} in the isomerised complex (24). Changed DNA structure within the isomerised σ^{54} -DNA complex

may also contribute to poor binding by core RNA polymerase. The strong reduction in isomerisation of the σ^{54} resulting from core RNA polymerase binding prior to exposure to activating conditions (Fig. 6A) and the weak binding of the isomerised σ^{54} to core RNA polymerase (Fig. 6B,C) is striking. It seems that normally, for efficient σ^{54} -holoenzyme isomerisation and open complex formation, activator dependent changes in σ^{54} structure would occur in concert with a changed binding of parts of σ^{54} to core RNA polymerase, but on the early melted DNA this is not occurring properly. As indicated above, when using the early melted DNA as template, the failure to re-configure interactions with DNA next to the promoter GC may simply strongly stabilise a σ^{54} conformation that is unfavourable for core RNA polymerase binding. We therefore suggest that in closed complexes activator drives a conformational change in σ^{54} that results in altered contacts to the early melted DNA (as detected in our KMnO_4 footprinting, see above) allowing a binding of σ^{54} and core RNA polymerase to permit full σ^{54} isomerisation and the associated isomerisation of the closed complex to the open complex. It seems that the Region I of σ^{54} greatly contributes to these events through (i) its requirement for creating the early melted DNA when holoenzyme binds homoduplex DNA, (ii) directing σ^{54} binding to the early melted DNA in heteroduplexes and associated fork junction structures, (iii) a binding interaction with core RNA polymerase and (iv) the changing Region I structure in isomerised σ^{54} and activated σ^{54} -holoenzyme (reviewed in 12). The recent demonstration that Region I sequences localise over the -12 promoter region is fully consistent with these observations and points to a central role of the protein and DNA elements that localise there in establishing new interactions that allow DNA melting and a changing binding relationship between core subunits and σ^{54} (41). We also note that the refractory behaviour of the σ^{54} -holoenzyme bound to the early melted DNA and the poor core binding of the isomerised σ^{54} are fully consistent with the view that interactions σ^{54} makes with the -12 promoter region, in particular sequences just downstream of the GC, are key in limiting spontaneous activator independent open complex formation (4).

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FOOTNOTES

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ABBREVIATIONS

DTT, dithiothreitol

PEG, polyethylene glycol

$\Delta\sigma^{54}$, deleted Region I σ^{54}

GTP, guanosine 5'-triphosphate

GTP γ S, guanosine 5'-*o*-(thiotriphosphate)

dGTP, 2'-deoxyguanosine 5'-triphosphate

GDP, guanosine 5' - diphosphate

NTP, nucleoside triphosphate

FIGURES.

Figure 1. Sequence of the *E. coli glnHp2* and *S. meliloti nifH* derivatives used in this work.

Compared to the original *glnHp2* m-12 promoter (27) sequence, heteroduplex promoter fragments contain either unpaired DNA from -11 to -10 with -10A (bottom strand) replaced with G to form a pre-melted structure comparable to that for the early melted *S. meliloti nifH* DNA (24) or from -11 to -6 (highlighted). In *glnHp2* m-12 wild type T:A base pair at -13 is replaced by G:C to increase sigma binding. *S. meliloti nifH* promoter fragments are as described previously (24).

Figure 2.

A. The supershifted isomerised σ^{54} -DNA complex forms in a reaction requiring activator and a hydrolysable nucleotide.

Gel shift assays were conducted with σ^{54} or $\Delta I\sigma^{54}$ (1 μ M), Region I (0.5 μ M), end-labelled *glnHp2* -11/-10 DNA (16 nM), PspF Δ H Δ TH activator (0.5 μ M) and dGTP (4 mM), where indicated. Sigma-DNA complexes were challenged with heparin (100 μ g/ml for 5 minutes) and loaded onto a native polyacrylamide gel, where bound and unbound DNA were separated.

B. The isomerised σ^{54} -DNA complex has an extended DNase I footprint.

Comparison of σ^{54} and isomerised complexes shows the footprint is extended by the action of activator. Removal of Region I prevents formation of the extended footprint. Reactions (10 μ l) were as in A except that *glnHp2* -11/-10 DNA was at 50 nM and PspF Δ H Δ TH at 4 μ M. Following exposure to DNase I bound and unbound DNA was separated on a native gel, eluted and analysed on a sequencing gel. Additions to each binding reaction are shown above each lane. Lanes 1 and 5, untreated DNA; lanes 2 and 6 DNase I cut DNA.

Figure 3. DNA melting by σ^{54} .

Reactions were as in 2B except that KMnO_4 replaced DNase I. Bound and unbound DNA was isolated from the native gel, cleaved with piperidine and analysed as in 2B. Sites of KMnO_4

reactivity are numbered. Additions to each binding reaction are shown above each lane. Lanes 1, untreated DNA; lanes 2, KMnO_4 treated DNA.

A. Template strand footprints. **B.** Non template strand footprints

Figure 4. σ^{54} -DNA complexes with *glnHp2* -11/-6 and -11/-10 promoter DNA have different gel mobilities.

Gel shift binding assay of σ^{54} with *glnHp2* DNA (16 nM) opened from -11 to -6, compared to -11/-10 opening. With *glnHp2* -11/-6 DNA a σ^{54} -DNA complex ($\text{ss}\sigma^*$ -DNA) is formed that has similar mobility to the activator and nucleotide dependent *glnHp2* -11/-10 supershifted complex ($\text{ss}\sigma$ -DNA) but reduced mobility compared to the σ -DNA complex. + indicates the presence of σ^{54} (1 μM), PspF Δ H Δ HTH (4 μM) and dGTP (4 mM). Heparin was added prior to gel loading. Results without heparin were similar.

Figure 5. Interaction of σ^{54} with -12 gapped DNA.

A. Gel mobility shift assay. Assays were conducted with σ^{54} or $\Delta\text{I}\sigma^{54}$ (1 μM), Region I (0.5 μM), end-labelled *glnHp2* -12 gapped duplex (100 nM), PspF Δ H Δ HTH (4 μM) and dGTP or GTP γ S (4 mM), where indicated (+).

B. KMnO_4 footprints. Assays were conducted as in A and then treated with KMnO_4 . Template strand footprints are shown and sites of KMnO_4 reactivity are numbered. Additions to each binding reaction are shown above each lane. Lane 1, untreated DNA; lane 2, KMnO_4 treated DNA.

Figure 6. Holoenzyme binding to *S. meliloti nifH* early melted DNA.

A. Gel mobility shift assay. Reactions contained *S. meliloti nifH* -12/-11 DNA (16 nM). Holoenzyme ($\text{E}\sigma^{54}$) was formed from 200 nM σ^{54} plus increasing amounts of core RNA polymerase (E) at 10, 25, 50, or 100 nM (indicated by triangle above lanes 3-6). dGTP (1 mM), PspF Δ H Δ HTH (4 μM) and σ^{54} (200 nM, lanes 1, 2) were added where indicated (+). Unactivated holoenzyme (100 nM E, 200 nM σ^{54}) DNA complex is shown in lane 7. After

binding, heparin (100 µg/ml) was added for 5 minutes. No new Eσ-DNA species is detected under activation conditions.

B. Core binding to the σ^{54} -DNA complex (graphed in C). A -35 to +6 bp *S. meliloti nifH* -12/-11 DNA fragment (500 nM, ref. 24) was used to form a supershifted complex (ss σ^{54} -DNA) with 300 nM σ^{54} , 1 mM GTP and 4 µM PspFΔH_{TH} (lane 2). GDP (10 mM) was added for 1 minute to inhibit activator function and then increasing amounts of core (E) were added (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 µM) for 5 minutes before gel loading (lanes 3-8). Lane 9 shows core (1.0 µM) binding to DNA. **C. Quantification of complexes from B (lanes 2-8).** ●, Eσ-DNA; □, ssσ-DNA; ■, σ-DNA. ,

Figure 7. Summary of footprint results.

The extents of DNase I protection, sites of KMnO₄ reactivity and protection are shown. DNase I protection, solid line; full (□) and partial (>) protection from KMnO₄ attack; *,*, enhanced reactivity in complexes forming with σ^{54} , its Region I deleted variant (ΔI σ^{54}) or in activator dependent σ^{54} isomerised complexes (ss σ^{54}).

Table 1. Quantification of KMnO₄ signals from figure 3.

KMnO₄ reactivities of T residues at positions -11, -9, -7 (template strand) and -8 and -6 (non template strand) in σ-DNA complexes are expressed by their ratio to signals without protein (reactivity = 1, lanes 2). Standard deviations are in brackets.

E. coli glnHp2 m-12

Homoduplex

-24 -12 +1
 -60.....TGCAAACT**GG**CACGATTTTT**GC**ATATATGTGAAT**T**GTCACGCAGGG.....+28
ACGTTTTGACCGTGCTAAAAACGTATATACACTTACAGTGCGTCCC.....

Heteroduplexes

-24 -12 +1
 -60.....TGCAAACT**GG**CACGATTTTT**GC****CA**ATATGTGAAT**T**GTCACGCAGGG.....+28
ACGTTTTGACCGTGCTAAAAACGT**G**TATACACTTACAGTGCGTCCC.....

-24 -12 +1
 -60.....TGCAAACT**GG**CACGATTTTT**GC****CACGCG**GTGAAT**T**GTCACGCAGGG.....+28
ACGTTTTGACCGTGCTAAAAACGT**G**TATACACTTACAGTGCGTCCC.....

S. meliloti nifH

Homoduplex

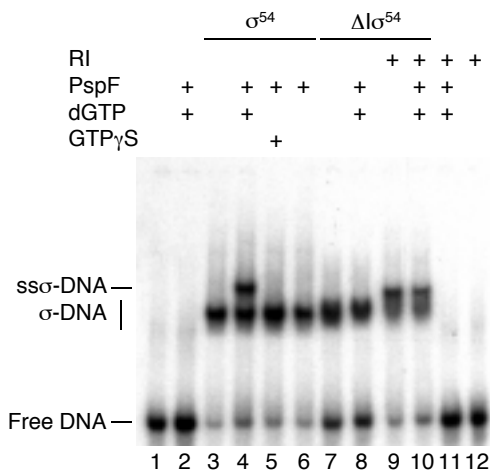
-25 -13 +1
 -60.....CAGACGGCT**GG**CACGACTTTTT**GC**ACGATCAGCCCT**GGG**CGCGCATG.....+28
GTCTGCCGACCGTGCTGAAAACGTGCTAGTCGGGACCCGCGCGTAC.....

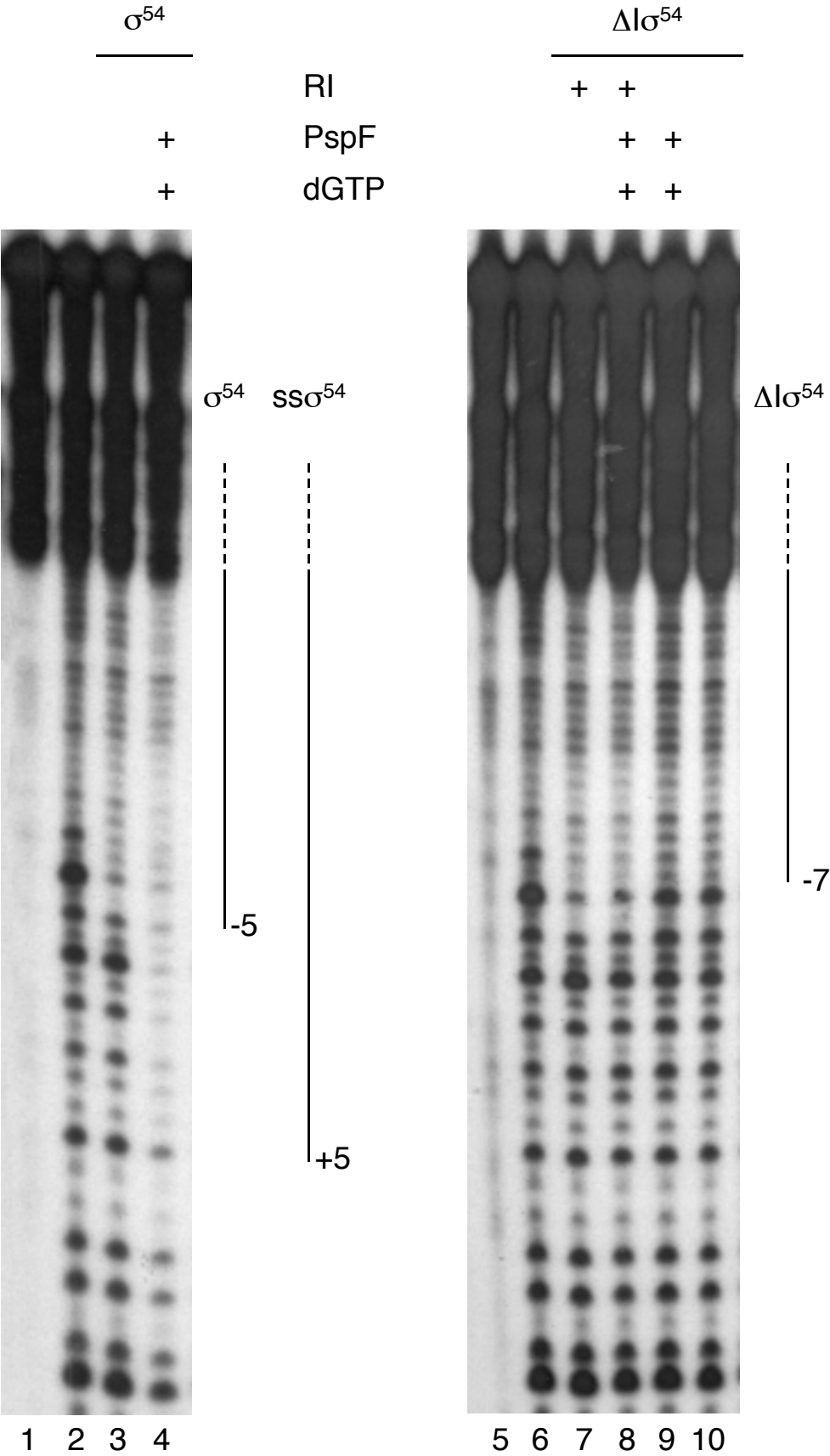
Heteroduplex

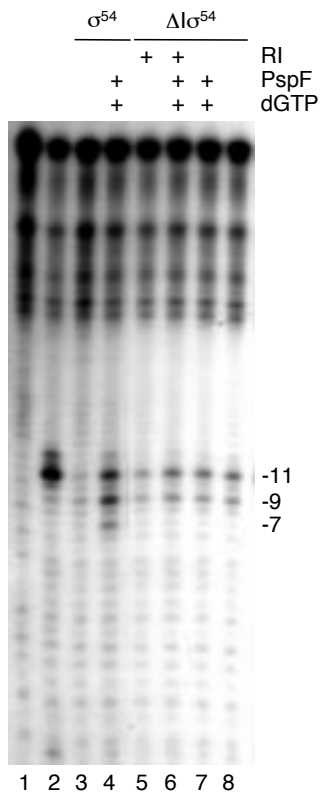
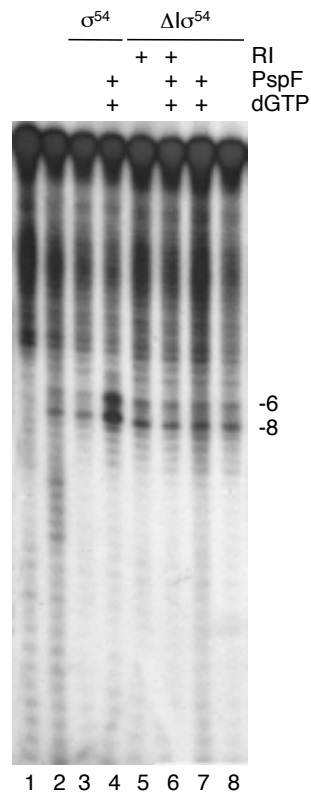
-25 -13 +1
 -60.....CAGACGGCT**GG**CACGACTTTTT**GC****CA**GATCAGCCCT**GGG**CGCGCATG.....+28
GTCTGCCGACCGTGCTGAAAACGTGCTAGTCGGGACCCGCGCGTAC.....

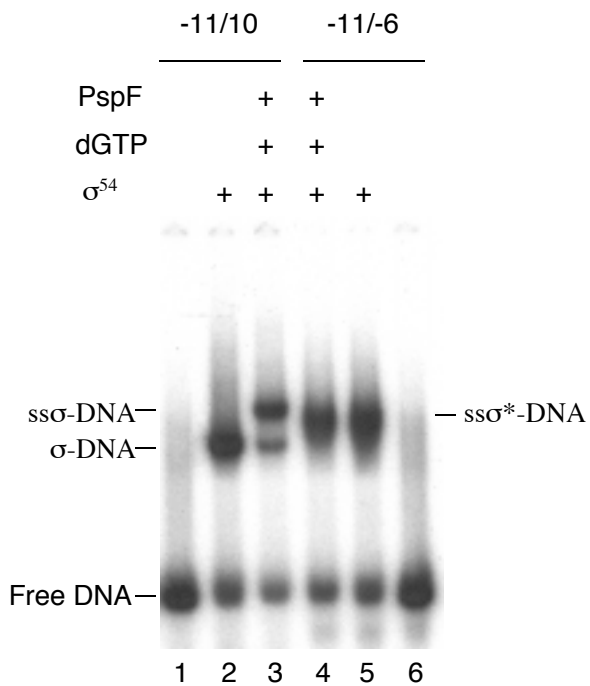
-12 gapped duplex

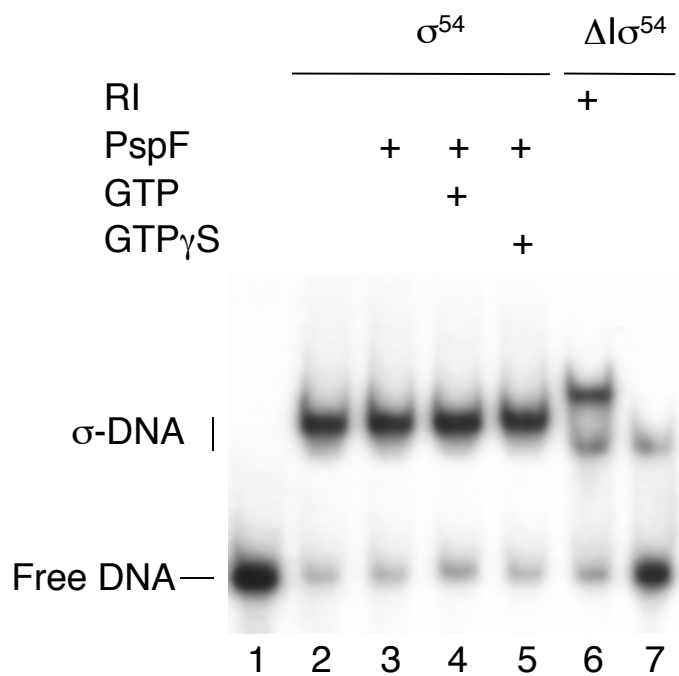
-25 -13 +1
 -60.....CAGACGGCT**GG**CACGACTTTTT**GC****█**CGATCAGCCCT**GGG**CGCGCATG.....+28
GTCTGCCGACCGTGCTGAAAACGTGCTAGTCGGGACCCGCGCGTAC.....



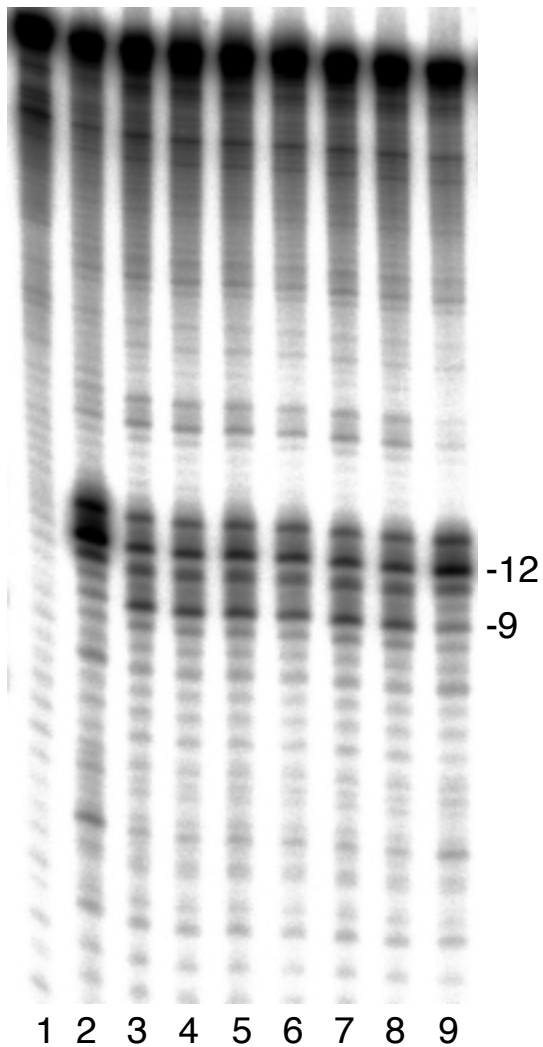


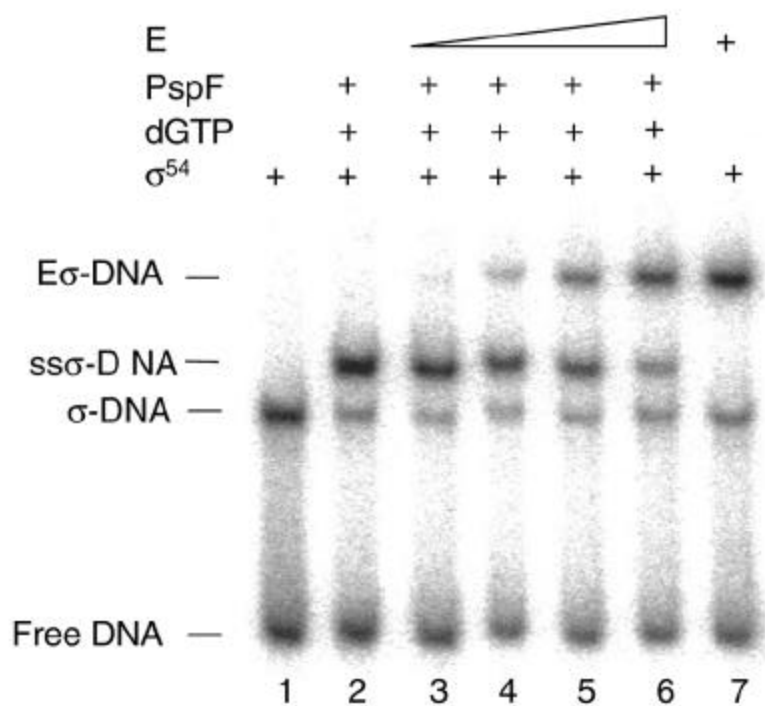
A**B**

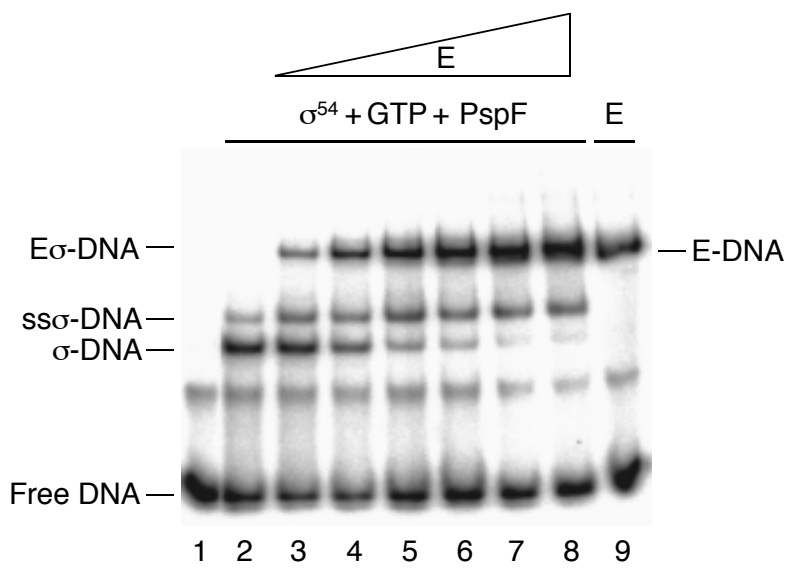


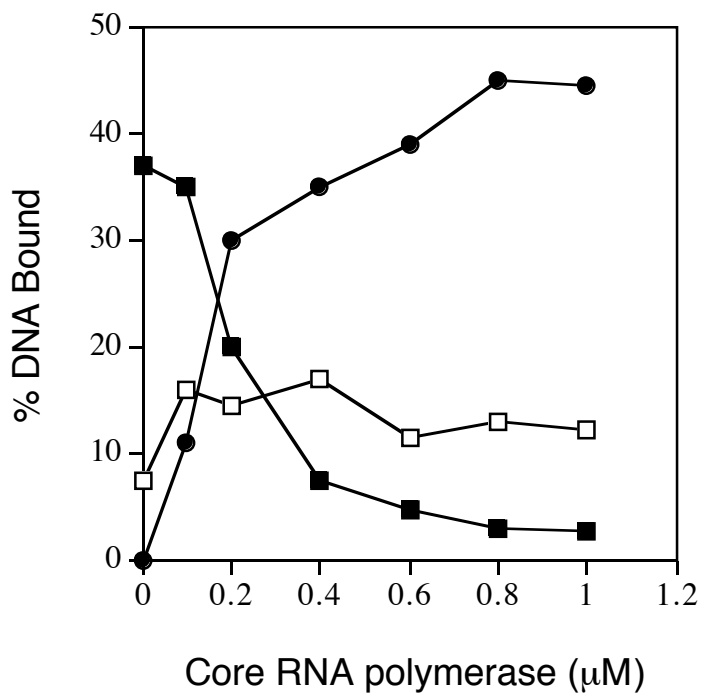


σ^{54}				$\Delta\sigma^{54}$	
				+	RI
				+	GDP
		+			dGTP
	+				GTP
+	+	+	+		PspF

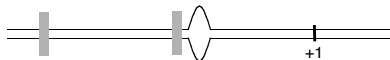






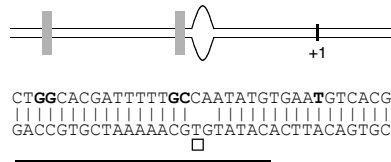


DNA



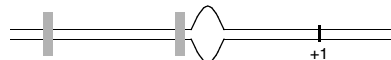
CTGGCACGATTTTTGCCAATATGTGAATGTCACG
GACCGTGCTAAAAACGTGTATACACTTACAGTGC
*

σ^{54}



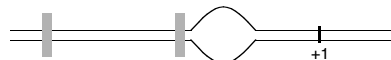
CTGGCACGATTTTTGCCAATATGTGAATGTCACG
GACCGTGCTAAAAACGTGTATACACTTACAGTGC
□

$\Delta\sigma^{54}$



CTGGCACGATTTTTGCCAATATGTGAATGTCACG
GACCGTGCTAAAAACGTGTATACACTTACAGTGC
■ *

σ^{54} + Activator + NTP
($ss\sigma^{54}$)



CTGGCACGATTTTTGCCAATATGTGAATGTCACG
GACCGTGCTAAAAACGTGTATACACTTACAGTGC
■ * *

T position	Strand	DNA	σ^{54} -DNA	ss σ^{54} -DNA	$\Delta I\sigma^{54}$ +RI	$\Delta I\sigma^{54}$
-11	template	1	0.14 (0.01)	0.44 (0.08)	0.27 (0.08)	0.36 (0.02)
-9	template	1	0.92 (0.03)	2.54 (0.37)	1.38 (0.47)	1.56 (0.14)
-8	non-template	1	0.80 (0.08)	2.09 (0.24)	0.99 (0.02)	0.98 (0.10)
-7	template	1	1.02 (0.03)	2.58 (0.17)	1.15 (0.50)	1.11 (0.20)
-6	non-template	1	0.69 (0.06)	1.90 (0.10)	0.96 (0.06)	0.92 (0.21)